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Original Article

Chemical composition, *in vitro* larvicidal and antileishmanial activities of the essential oil from *Citrus reticulata* Blanco fruit peel

Composição química, atividades larvicida e leishmanicida *in vitro* do óleo essencial da casca do fruto de *Citrus reticulata* Blanco

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Abstract

Numerous studies have investigated the chemical composition and biological activities of essential oils from different *Citrus* species fruit peel, leaves and flowers. This paper aims to investigate the chemical composition, larvicidal and antileishmanial activities of essential oil from *Citrus reticulata* fruit peel (CR-EO). CR-EO was obtained by hydrodistillation in a Clevenger-type apparatus and its chemical composition was analyzed by GC-MS and GC-FID. Limonene (85.7%), γ-terpinene (6.7%) and myrcene (2.1%) were identified as its major components. CR-EO showed high activity against promastigote forms of *Leishmania amazonensis* (IC_{50} = 8.23 µg/mL). CR-EO also exhibited high larvicidal activity against third instar *Aedes aegypti* larvae at a lethal concentration (LC_{50} = 58.35 µg/mL) and 100% mortality at 150 µg/mL. This study suggests, for the first time, the potential use of CR-EO against this important mosquito-borne viral disease caused by the genus *Aedes*.

Keywords: *Leishmania amazonensis*, *Aedes aegypti*, limonene.

Resumo

Numerosos estudos têm investigado a composição química e as atividades biológicas de óleos essenciais extraídos de cascas dos frutos, folhas e flores de diferentes espécies de *Citrus*. Este trabalho tem como objetivo investigar a composição química e as atividades larvicida e leishmanicida *in vitro* do óleo essencial das cascas dos frutos de *Citrus reticulata* (CR-EO). CR-EO foi obtido pela técnica de extração em aparelho Clevenger e sua composição química foi determinada por CG-EM e CG-DIC. Limoneno (85,7%), γ-terpineno (6,7%) and mirceno (2,1%) foram identificados como os constituintes majoritários. CR-EO mostrou alta atividade contra as formas promastigota de *Leishmania amazonensis* (CI_{50} = 8,23 µg/mL). CR-EO também exibiu alta atividade larvicida contra as larvas do terceiro estágio do *Aedes aegypti* com concentração letal (CL_{50} = 58,35 µg/mL) e mortalidade de 100% em 150 µg/mL. Este estudo sugere, pela primeira vez, o uso potencial de CR-EO contra esta importante doença viral transmitida por mosquitos do gênero *Aedes*.

Palavras-chave: *Leishmania amazonensis*, *Aedes aegypti*, limoneno.

1. Introduction

Concern for the control and fight against *Aedes aegypti*, the mosquito that is the main vector of a severe hemorrhagic disease known as dengue, has been latent (Wankhar et al., 2015). The number of dengue cases has reached about 400 million per year. In the last 50 years, this disease has been endemic in 128 countries, while 36 countries are considered dengue-free ones. The highest prevalences are found in countries in the Americas, Asia and Africa, i. e., 3.97 billion inhabitants are exposed to the risk of infection (Brady et al., 2012). In addition to the dengue virus, the vector may transmit other diseases known all over the world as chikungunya and zika (Neves et al., 2017).

Leishmaniasis is another worrisome disease worldwide which is also caused by the infected vector known as "straw mosquito" (Silva et al., 2020). The estimate is that 2 million new cases of leishmaniasis occur annually and that between 15 and 20 million people have got the disease in the world (Moreira et al., 2019). In the search for plants

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with therapeutic potential, species of the genus *Citrus* not only have economic importance, but also produce bioactive essential oils (EOs) which have high value in perfume, food and beverage industries (Dosoky and Setzer, 2018).

EOs and extracts from *Citrus reticulata* fruit peel and leaves have several biological applications, such as antimicrobial, antioxidant, anti-inflammatory, anticancer, antiproliferative, anti-pulmonary fibrosis, hypoglycemic and insecticidal ones, besides being useful in skin care (Hamdan et al., 2016; Apraj and Pandita, 2016). Therefore, this study aimed to evaluate the chemical composition, larvicidal and anti-*Leishmania amazonensis* activities of essential oil from *C. reticulata* fruit peel (CR-EO). So far, larvicidal activity of CR-EO against third instar *A. aegypti* larvae has not been investigated in the literature.

2. Material and Methods

2.1. Plant material

Fruits were collected in Rio Verde (17°99.4'63.2"S and 51°05.2'44.6"W), a city located in Goiás state, Brazil, on January 18th, 2019, at 9 a.m. The plant was identified by the botanist Luzia Francisca de Souza and a voucher specimen of *Citrus reticulata* was deposited in the herbarium in Rio Verde, at the Instituto Federal Goiano (IFGOIANO) under identification number #4488.

2.2. Essential oil extraction

Essential oil (EO) was extracted from *C. reticulata* (CR-EO) fruit peel by hydrodistillation for 3 h in a Clevenger-type apparatus. Hydrodistillation was performed in triplicate. To this end, fruit peel was divided into three 500-g samples and 500 mL distilled water was added to each sample. After manual collection of EO, remaining traces of water were removed with anhydrous sodium sulfate. Filtration was then carried out. EO was stored in an amber bottle and kept in a refrigerator at 4 °C until analysis (Carneiro et al., 2017).

2.3. Chemical identification of essential oil from C. reticulata fruit peel (CR-EO)

CR-EO was dissolved in ethyl ether and analyzed by gas chromatography-flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC–MS), with the use of Shimadzu QP5000 Plus and GCMS2010 Plus (Shimadzu Corporation, Kyoto, Japan) systems. The temperature of the column in GC-FID was programmed to rise from 60 to 240 °C at 3 °C/min and was held at 240 °C for 5 min; the carrier gas was H_2 at a flow rate of 1.0 mL/min. The equipment was set to operate in the injection mode; the injection volume was 0.1 µL (split ratio of 1:10), while injector and detector temperatures were 240 and 280 °C, respectively. Relative concentrations of components were obtained by normalizing peak areas (%). Relative areas consisted of the average of triplicate GC-FID analyses. GC-MS conditions and the identification of essential oils have been previously reported (Lemes et al., 2018). Identification of volatile components of essential oil from *C. reticulata* (Table 1) was based on their retention indices on an Rtx-5MS (30 m × 0.25 mm; 0.250 µm) capillary column under the operating conditions used for GC relative to a homologous series of *n*-alkanes (C_8-C_{20}). Structures were computer-matched with Wiley 7, NIST 08, and FFNSC 1.2, and their fragmentation patterns were compared with literature data (Adams, 2007).

Table 1. Chemical composition of essential oil from *Citrus reticulata* fruit peel (CR-EO).

RTmin	Compounds	RI_{exp}	RI_{lit}	%RA
6.28	α-Thujene	852	851	0.2
9.09	α-Pinene	935	934	0.6
10.78	Sabinene	975	974	0.5
11.57	**Myrcene**	993	991	**2.1**
13.82	**Limonene**	1039	1039	**85.7**
14.32	*Trans*-β-ocimene	1051	1050	0.7
14.81	γ-terpinene	1062	1062	**6.7**
18.59	Limonene oxide	1132	1133	0.5
19.31	Citronellal	1158	1158	0.1
21.13	α-Terpineol	1184	1185	0.1
24.12	Geraniol	1254	1255	0.1
24.86	Geranial	1271	1270	0.2
37.92	Caryophyllene oxide	1581	1581	0.3
	Total			97.8

RTmin: retention time; **RI_{exp}**: Retention index relative to *n*-alkanes (C_8–C_{20}) on the Rtx-5MS column; **RI_{lit}:** Kovats retention index (values from literature – Adams, 2007); **%RA:** Relative abundance.

2.4. Larvicidal assay

Larvae of *A. aegypti* were obtained from the Laboratório de Patologia Tropical e Saúde Pública that belongs to the Universidade Federal de Goiás, UFG, Brazil. The larvicidal assay was performed in agreement with a previously reported method (Mesquita et al., 2018), as follows: larvae were kept in plastic trays under controlled temperature (26 ± 2 °C) and humidity (70-80%) until they reached the third final instar stage. Afterwards, 10 larvae were transferred to 50-mL plastic cups, each containing 10 mL mineral water and ground fish food (TetraMin Tropical Flakes), followed by the addition of 100 µL solution of CR-EO in dimethyl sulfoxide (DMSO) (25-500 µg/mL). After 24 hours, the number of dead larvae was counted and the lethal percentage was calculated. All experiments were carried out in quintuplicate, including a negative control treatment with DMSO, mineral water, larvae and ground fish food. Permethrin was used as a positive control. Larvicidal activities were reported as lethal concentration at 50% (LC_{50}), representing the concentration in micrograms per milliliter that caused 50% larval mortality, with 95% confidence interval. Mortality data were assessed by probit analysis (Finney, 1971). Mortality data were treated by the Polo plus® software (Robertson et al., 2003) with 95% confidence interval and values of $P < 0.05$ were considered statistically significant.

Table 2. Antileishmanial activity of essential oil from *Citrus reticulata* fruit peel (CR-EO).

	Concentrations (μg/mL) ± Standard deviation					
	100	**50**	**25**	**12.5**	**6.25**	**IC_{50} (μg/mL)**
CR-EO	100 ± 0.00	100 ± 0.00	64.86 ± 5.98	58.14 ± 7.69	47.92 ± 4.86	8.23 ± 1.10
	0.19	**0.095**	**0.047**	**0.023**	**0.011**	
Amph. B*	44.38 ± 0.53	36.89 ± 0.79	33.61 ± 0.62	29.02 ± 1.85	23.50 ± 1.58	0.25 ± 0.39

*Positive control: Amphotericin B 1μg/mL; Negative control: Medium RPMI + 0.1% DMSO; IC_{50} (average inhibitory concentration).

2.5. Antileishmanial assay

To evaluate antileishmanial activity, *L. amazonensis* promastigote forms (MHOM/BR/PH8) were maintained in RPMI 1640 (Gibco) culture medium supplemented with 10% fetal bovine serum, penicillin (100 UI/mL) and streptomycin (100 μg/mL). Subsequently, about 1 x 10^6 parasites were distributed on 96-well plates, while CR-EO previously dissolved in 100% dimethylsulfoxide (DMSO, stock solution 100 mM) (Synth) was added to the cultures at concentrations of 6.25 to 100 μg/mL. Amphotericin B (Sigma Aldrich, 97% purity), at concentrations ranging from 0.011 to 0.19 μg/mL, was added to cultures and used as positive control. Cultures were incubated in a BOD (Quimis) incubator at 25 °C for 24 h and antileishmanial activity was determined by verifying whether growth of promastigote forms was inhibited. It was carried out by counting the total number of live promastigotes in the Neubauer (Global Glass - Porto Alegre, BR) chamber on the basis of flagellar motility. RPMI 1640 medium (Gibco) containing 0.1% DMSO (Synth) (highest concentration) was used. Results were expressed as the average of the percentage of growth inhibition relative to the negative control (0.1% DMSO) (Cabral et al., 2020).

3. Results and Discussion

Volatile compounds were identified by gas chromatography-flame ionization detection (GC-FID) and gas chromatography–mass spectrometry (GC-MS). Thirteen compounds, which represent 97.8% of all components, were identified in the oil from fruit peel. Major compounds found in CR-EO were limonene (85.7%), γ-terpinene (6.7%) and myrcene (2.1%) (Table 1).

Previous reports of EOs from fruit peel from other *C. reticulata* specimens showed that terpenes limonene, sabinene, linalool, γ-terpinene, octanal and capraldehyde were its major constituents (Hamdan et al., 2016; Martins et al., 2017). EOs from different *Citrus* species are chemically similar with the predominance of the monoterpene limonene, characteristics that are pointed out by Bozkurt et al. (2017). High limonene concentration (74.38%) was also identified in *C. reticulata* peel grown in Spain (Espina et al., 2011). A recent study of ten *Citrus* species should be highlighted, since it reinforces limonene and aldehyde compounds in CR-EO (González-Mas et al., 2019).

CR-EO had its larvicidal activity investigated against third instar *A. aegypti* larvae. Initially, larvae in contact with CR-EO showed accelerated movement. However, after prolonged exposure, they began to exhibit tremor and slow or lethargic movement, even when artificially stimulated. CR-EO also caused darkening of the entire larval body. Doses of 12.5, 25, 50 and 100 μg/mL resulted in 10.2, 25.1, 51.7 and 85.3% of dead larvae, respectively, while 150 μg/mL ensured 100% mortality. LC_{50} of CR-EO was 58.35 μg/mL. Even though EOs from several *Citrus* species have already been tested against *A. aegypti* larvae, this is the first promising report of *C. reticulata*. Larvicidal and insecticidal activities have already been evaluated in EOs from *C. sinensis, C. limon, C. grandis, C. aurantifolia, C. hystrix, C. maxima* and *C. medica* (Araújo et al., 2016; Gomes et al., 2019; Sarma et al., 2017, 2019; Soonwera, 2015). High larvicidal activity could be due to the high limonene concentration (85.7%), a botanical insecticide which has been patented as an active agent of larvicidal formulations (Dias and Moraes, 2014).

Regarding antileishmanial activity, CR-EO was promising against promastigote forms of *L. amazonensis* (IC_{50} = 8.23 μg/mL) (Table 2).

Several authors have reported that EOs whose values are IC_{50} < 10 μg/mL are highly active (Silva et al., 2020; Almeida et al., 2020). The range of IC_{50} values that considers EOs highly active has been described by Estevam et al. (2016), who studied EOs from C. *limonia* and *C. latifolia* fresh leaves. It should be highlighted that results found by the study reported by this paper do not agree with the ones reported by Monzote et al. (2019), since the IC_{50} value of commercially obtained CR-EO was 70.7 μg/mL. This fact leads to questioning the nature of EOs that are commercialized with no compliance with regulations. On the other hand, high antileishmanial activity of CR-EO found by this study may also be justified by the amount of limonene, a monoterpene that exhibits this activity against parasites that belong to the genus *Leishmania* (Arruda et al., 2009).

4. Conclusion

This study described the chemical composition of CR-EO, in which thirteen compounds were identified, and showed their high larvicidal activity against *Aedes aegypti* larvae and anti-*Leishmania amazonensis* promastigote forms. Limonene, as the major compound in CR-EO, could explain their high larvicidal and antileishmanial activities. This communication is the first report of larvicidal evaluation of CR-EO.

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